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Reverse Transcriptase Genotype and Antiretroviral Susceptibility of Human Immunodeficiency Virus Isolates From Patients With Advanced Disease Treated With Didanosine: Correlation With Virologic Response and Survival

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To identify correlates of virologic response and survival, the reverse transcriptase (RT) genotype and in vitro antiviral susceptibility of human immunodeficiency virus (HIV) isolates from 20 patients treated with didanosine were studied. Patients had advanced HIV disease and were intolerant to or had failed zidovudine. Neither RT genotype nor antiviral susceptibility testing, as determined by a peripheral blood mononuclear cell–based assay, correlated with a virologic response to didanosine, as determined previously by quantitative serum culture. Only one (8%) of 12 isolates obtained after 6–12 months of treatment showed mutation at codon 74 conferring didanosine resistance. Reversions were seen in three of five patients with pre-treatment zidovudine resistance mutations at codons 70, but in none of eight with mutations at codon 215. Pretreatment isolates encoding mutations at RT codon 215 or encoding codon 123 asp were associated with both significantly greater CD4 lymphocyte depletion and shorter survival. In this cohort of patients with advanced HIV disease, neither rapid emergence of didanosine resistance nor rapid reversion of zidovudine resistance was observed. To better understand the relationship between virologic response and in vitro susceptibility to didanosine, more precise tools may be needed.

KEY WORDS: AIDS, resistance, zidovudine, AZT, ddI

INTRODUCTION

Patients with advanced human immunodeficiency virus (HIV) disease experiencing intolerance to or treatment failure during zidovudine therapy may derive clinical and virologic benefit when changed to didanosine [Yarchoan et al., 1990; Lambert et al., 1990; Shepp and Ashraf, 1993; Spruance et al., 1994]. In a previous study, a virologic response to this change in antiretroviral therapy was shown to correlate with short-term clinical effects indicated by change in body weight, but not with survival [Shepp and Ashraf, 1993]. In this study, the relationship between the antiviral drug resistance phenotype, reverse transcriptase (RT) genotype, virologic response and survival during didanosine treatment was examined.

METHODS

Patients

This research was approved by the Research, Clinical Investigations and Publications Committee of North Shore University Hospital. All participants gave written informed consent prior to entry. The study included the 21 patients from a previous study from whom isolation of HIV from serum was successful. The characteristics of the patient population have been reported [Shepp and Ashraf, 1993]. All had advanced HIV disease and were either intolerant to or had disease progression while receiving zidovudine. Didanosine treatment was initiated under the expanded access program. Twenty patients were followed clinically until death and one patient was alive as of March 1995.

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HIV Isolates

Quantitative HIV cultures were performed using serial dilutions of patient serum and phytohemagglutinin (PHA)-stimulated normal donor peripheral blood mononuclear cells (PBMC), as previously described [Shepp and Ashraf, 1993]. After 10 to 14 days of culture, supernatants were mixed with an equal volume of fetal bovine serum (FBS), aliquoted and stored at −80°C. Specimens for culture were obtained before treatment and after 2, 6 and 12 months. A virologic response to treatment was defined as a fivefold or greater decrease in titer when the pre-treatment and the 2 month quantitative cultures were compared. For each patient, the last available isolate, at either 6 or 12 months, was considered the post-treatment isolate.

Antiviral Susceptibility Testing

The titer of HIV stocks were determined by culturing serial tenfold dilutions in 2×10^6 normal donor PHA-stimulated PBMC in duplicate wells of a 24 well tissue culture plate. Cultures were grown in RPMI 1640 with 20% FBS, 10% T-cell growth factor (Cellular Products, Buffalo, NY), and DEAE-Dextran 8 μg/ml (medium). Cultures were refed with fresh medium twice weekly. After 14 days, the presence of HIV infection was determined by detection of HIV p24 antigen in culture supernatant using a commercially available ELISA (Coulter Immunology, Hialeah, FL), and the 50% tissue culture infectious dose ($TCID_{50}$) was calculated. Stocks with insufficient titer for susceptibility testing were regrown once and retitered.

Bulk cultures of PHA-stimulated PBMC were infected with the test isolate at 10 $TCID_{50}/2 \times 10^6$ cells. After 2 hours, cultures were washed three times with phosphate buffered saline and 2×10^6 infected PBMC were placed into each well of a 24 well culture plate and cultured in duplicate with medium plus drug at concentrations of 0, 0.01, 0.1, 1.0, 10. 100 μM for zidovudine (provided as a gift by Catherine Patishall, Burroughs Wellcome Co., Research Triangle Park, NC) and 0, 0.05, 0.5, 5.0, 50, 500 μM for didanosine (provided as a gift by Anthony Razel, Bristol Myers-Squibb, Inc., Wallingford, CT). Plates were incubated at 37°C in 5% CO_2 atmosphere and cultures were refed twice weekly with medium plus drug at the appropriate concentrations. After 14 days, HIV p24 antigen was quantitated in serial tenfold dilutions of culture supernatant, the concentration of antigen plotted against drug concentration and the 90% inhibitory concentrations (IC_{90}) for zidovudine and didanosine determined. In this assay, IC_{90} values were found to give results comparable to IC_{50} values as determined by assays employing higher viral inocula [Japour et al., 1993]. Paired isolates from the same patient were tested in parallel.

Reverse Transcriptase Genotyping

Using a phenol/chloroform method, DNA was extracted from infected cell pellets prepared at the end of the HIV stock titration experiments. A 738 nucleotide segment from the HIV RT gene was then amplified using nested polymerase chain reaction (PCR). The following primers were used: RT-0 5′-CCCATTAGTCCTATTGAAACTG; RT-3 5′-GCAGCATTATAGGCTGTACTGT; RT-1 5′-ACTGTACCAGTAAAATTAAAGCC; RT-2 5′-TGTCCATTTATCAGGATGGAGTT.

Sequences of the primers correspond to nucleotides 2104–2125, 2820–2841, 2122–2144, and 2800–2822, respectively, of the HIV pol gene [Sanchez-Pescador et al., 1985]. The internal primer pair flanks codons 15–232 of the RT gene. The initial PCR reaction mixture contained 0.5 μg DNA, 0.8 μM primers RT-0 and RT-3, 400 μM each of four deoxynucleoside triphosphates, 4 mM $MgCl_2$, 2.5 units Taq polymerase in PCR buffer (GeneAmp PCR Reagents, Perkin-Elmer Cetus, Norwalk, CT). The PCR reaction was carried out on a Perkin-Elmer model 9600 thermal cycler set as follows: denaturation at 94°C for 60 seconds, annealing at 50°C for 90 seconds, extension at 72°C for 120 seconds, 35 cycles. The final cycle was followed by autoextension for 10 minutes at 72°C. The conditions for the PCR reaction with primers RT-1 and RT-2 were identical except 1 μl of the product of the initial PCR reaction was utilized and annealing was performed at 52°C. PCR products were column purified (Wizard PCR Preps, Promega, Madison, WI) and purity of PCR products was confirmed visually by agarose gel electrophoresis and ethidium bromide staining.

DNA sequence was determined by the cycle sequencing method [Lee et al., 1992] using both RT-1 and RT-2 as template primers, dye-labeled dideoxynucleotide chain terminators, and an automated DNA sequencer (model 373A, Applied Biosystems, Foster City, CA). DNA sequences and the predicted amino acid sequences were compared to the HIV reference strain ARV-2 [Sanchez-Pescador et al., 1985].

Statistical Analysis

Measured data were compared by Wilcoxon rank-sum test or Student's t-test and categorical data by Fisher's exact test. All tests of significance were two-tailed.

RESULTS

The stored viral stock of the HIV isolate from one patient was insufficient for testing. Therefore, 20 patients were studied. RT genotyping was performed on all 20 pre-treatment isolates. Fifteen of these isolates also had sufficient titer for antiviral susceptibility testing. Twelve post-treatment isolates were obtained during therapy (six each at 6 and 12 months) and all had RT genotype determined. Eleven also had sufficient titer for susceptibility testing. At the start of didanosine therapy, the median CD4 count for the 20 patients was 18/mm^3 (interquartile range 8–32); nine patients were clinically staged as CDC group C and 11 as CDC group B3 [CDC, 1992]. The median duration of didanosine therapy was 32 weeks (interquartile range 22–61).

RT Genotyping

None of 20 pre-treatment isolates had mutations encoding amino acid substitutions at codons 65 [Zhang et

TABLE I. RT Gene Sequence Variation and In Vitro Susceptibility to Didanosine

Patient	Codon	Nucleotide sequence		Amino acid		Didanosine IC_{90} (μM)	
		Pre	Post	Pre	Post	Pre	Post
9	74	TTA	GTA	Leu	Val	0.05	1.0
12	135	GTC	GTA	Val	Val	1.0	0.9
14	135	ATA	ACA	Ile	Thr	1.0	4.0
15	122	AAA	GAA	Lys	Glu	0.1	4.0
	215	ACC	TAC	Thr	Tyr		
	221	CAT	GAT	His	Asp		
	226	GCA	GGA	Ala	Gly		

TABLE II. Unusual RT Gene Codon 215 Mutations Identified in HIV Isolates From Patients Treated With Didanosine

Patient	Nucleotide sequence		Amino acid		Zidovudine IC_{90} (μM)	
	Pre	Post	Pre	Post	Pre	Post
3	GAC	TAC	Asp	Tyr	ND	ND
1	ATC/ACC[a]	ND	Ile/Thr[a]	ND	ND	ND
6	TAC/CAC[a]	ND	Tyr/His[a]	ND	ND	ND
4	TGC	ND	Cys	ND	15	ND
17	CAG	ND	Gln	ND	9	ND
9	ACC	TAC	Thr	Tyr	0.8	2.0
15	ACC	TAC	Thr	Tyr	0.2	90

[a]Approximately equal mixture of sequences was identified.
ND = not done.

al., 1994; Gu et al., 1994], 74 [St. Clair et al., 1991], 75 [Lacey and Larder, 1994], or 184 [Gu et al., 1992], previously reported to confer resistance to didanosine. One pre-treatment isolate had a mutation at codon 135 (Table I, patient 12) previously associated with didanosine resistance [Gao et al., 1992]. Mutation at codon 74 conferring didanosine resistance was identified in only one of 12 (8.3%) post-treatment isolates (Table I, patient 9). Emergence of mutation at codon 135 also was identified in one post-treatment isolate (Table I, patient 14). Mutations at codons 65, 75, or 184 were not seen.

Sixteen of 20 pre-treatment isolates had mutations conferring coding changes at one or more of five codons (41, 67, 70, 215, 219) resulting in zidovudine resistance [Larder and Kemp, 1989; Kellam et al., 1992]. Fourteen had mutations at codon 215. Eight of these 14 isolates had mutations encoding tyrosine, including one mixture of genotypes (Table II, patient 6), and two had mutations encoding phenylalanine. Four other isolates had sequences encoding unusual sequences (Table II, patients 1, 3, 4, 17); one was a mixture. The median duration of prior zidovudine therapy was 16.5 months for patients with codon 215 mutations as compared to 6.0 months for those with wild-type sequences (Wilcoxon rank-sum test, $P = 0.06$). Ten pre-treatment isolates had coding mutations at codon 41 and seven had three or more zidovudine resistance mutations.

Paired isolates from two patients showed acquisition of the mutation thr 215 tyr and a third showed development of asp 215 tyr (Table II, patients 9, 15, 3) during didanosine treatment. Two of these patients had previously experienced severe hematologic toxicity during zidovudine administration and none of the three showed elevation of the mean corpuscular volume or any severe hematologic toxicity during the study.

Among the nine patients with paired isolates and any zidovudine resistance mutations in the pre-treatment isolate, reversion of one or more mutations was seen in six (3 at 6 months and 3 at 12 months). Reversion occurred in 3 of 5 patients with mutations at codon 70, 0 of 5 with mutations at codon 67, 2 of 6 with mutations at codon 219, 2 of 5 with mutations at codon 41 and 0 of 8 with mutations at codon 215. Two of three reversions at codon 70 resulted in a change in sequence from AGA encoding arginine to GGA encoding glycine, rather than to the wild-type sequence AAA encoding lysine, as has been noted previously [Smith et al., 1994]. The two reversions at codon 219 involved changes in sequence from CAA encoding glutamine to CCA encoding proline and CTA encoding leucine, rather than AAA encoding lysine.

Susceptibility Testing

The results of susceptibility testing for pre- and post-treatment isolates are shown in Figure 1. The pre- and post-treatment geometric mean IC_{90}s for didanosine were 0.49 μM (95% confidence interval [CI] 0.20–1.2) and 0.67 μM (95% CI 0.22–2.0), respectively. The pre- and post-treatment geometric mean IC_{90}s for zidovudine were 3.3 μM (95% CI 0.82–13) and 4.6 μM (95% CI 1.0–21), respectively. All seven isolates (from five patients) encoding threonine at codon 215 had IC_{90}s for zidovudine below 1 μM and 17 of 19 isolates (from 12 patients) encoding mutations at codon 215 had IC_{90}s for zidovudine above 1 μM, while two (from one patient) were lower. In one patient, high level resistance to zidovudine appeared in the post-treatment isolate in association with the development of mutation at codon 215 (Table II, patient 15), while in another, the IC_{90} declined 20-fold in association with loss of mutations at codons 70 and 219.

Paired isolates from two patients showed reductions in sensitivity to didanosine of greater than one log. One isolate pair showed a 20-fold reduction in sensitivity associated with acquisition of the leu 74 val mutation and in the other, a 40-fold reduction was associated with development of four mutations (Table I, patients 9, 15). In a third patient, the IC_{90} for didanosine fell by 80-fold. For all other isolate pairs, the pre- and post-treatment sensitivities to both drugs differed by fivefold or less.

Correlation With Virologic Response

The pre-treatment geometric mean IC_{90} for didanosine was 0.8 μM (95% CI 0.4–1.7) in the 14 patients who had a virologic response to didanosine treatment and 0.25 μM (95% CI 0.04–1.8) in the six who did not. The pre-treatment geometric mean IC_{90} for zidovudine was 2.1 μM (95% CI 0.3–14) and 7.9 (95% CI 0.1–65) in responders and non-responders. A virologic response to treatment was seen in 9/14 (64%) patients with and 5/6 (83%) without codon 215 mutations in the pre-treatment iso-

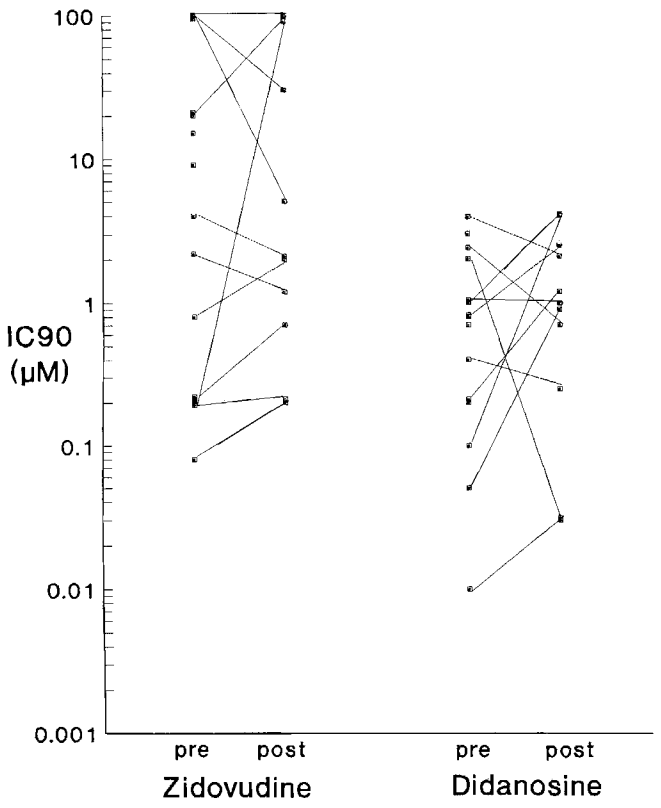


Fig. 1. Antiviral susceptibility testing of isolates obtained from patients before (n = 15) and 6–12 months after (n = 11) initiation of didanosine treatment.

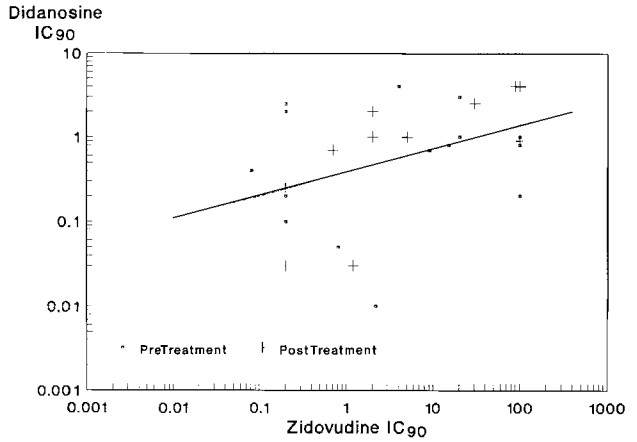


Fig. 2. Correlation between IC_{90} values for zidovudine and didanosine among 26 pre- and post-didanosine treatment isolates (r = 0.41; P = 0.04).

late (P = 0.09, Fisher's exact test). A modest correlation was observed between the zidovudine and didanosine IC_{90} values obtained for each isolate (Fig. 2).

In an attempt to identify a relationship between RT genotype and virologic response, eight codons (25, 122, 123, 128, 174, 196, 207, 211) showing sequence variation in five or more pre-treatment isolates and mutations encoding amino acid substitutions appearing in post-treatment isolates were compared in responders and non-responders. No genotypic patterns or novel mutations could be correlated with virologic response.

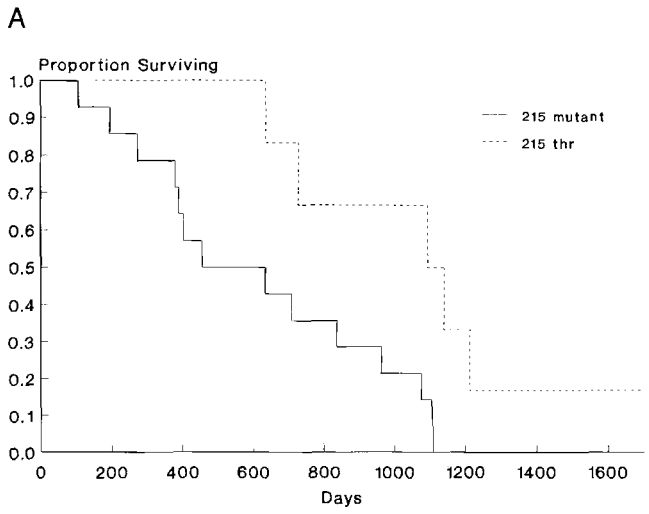
Correlation With Survival

Figure 3 shows survival in patients with or without mutations in the pre-treatment isolate at codons conferring zidovudine resistance. The presence of codon 215 mutation in the pre-treatment isolate was associated with a median survival of 543 days compared to 1,116 days when wild-type sequences were found (P = 0.016, Wilcoxon rank-sum test). The presence of codon 215 mutation was also strongly associated with lower CD4 lymphocyte counts, another factor correlating with poor survival. The median CD4 lymphocyte count at the start of treatment in patients with mutations at codon 215 was 11/mm^3, compared to 134/mm^3 in those without mutations (Wilcoxon rank-sum test, P = 0.0016). The presence of codon 41 mutation was not associated with any alteration of survival.

The effect of sequence variation at the eight codons listed above on survival was also studied. At codon 123, 11 pre-treatment isolates had the sequence GAC encoding aspartic acid found in the HIV reference strain [Sanchez-Pescador et al., 1985]. Five isolates had the sequence GAA encoding glutamic acid and four had GGA encoding glycine. The median survival was 401 days in patients with isolates encoding 123 asp compared to 1,093 days in patients with isolates encoding 123 glu or gly (Fig. 4; Wilcoxon rank-sum test, P = 0.007). Analysis of the seven other codons did not reveal any correlation with survival. Analysis of post-treatment isolates showed that codon 123 sequences were stable in eight of 11 pairs, while three changed from glycine to aspartic acid during treatment. None changed from aspartic acid to glycine or glutamic acid. The presence of aspartic acid at codon 123 in the pre-treatment isolate was strongly associated with other factors affecting survival. The median CD4 lymphocyte count in patients with sequences encoding 123 asp was 11/mm^3 (range 2–22) and 34 (range 7–193) in those with codon 123 glu or gly (Wilcoxon rank-sum test, P = 0.009). Ten (91%) of 11 isolates with codon 123 asp also had codon 215 mutations compared with 4 (44%) of 9 with codon 123 glu or gly (Fisher's exact test, P = 0.05). For isolates with codon 123 asp, the geometric mean IC_{90} value for didanosine was higher than for those with codon 123 glu or gly (1.0 μM vs. 0.25 μM; Student's t-test, P = 0.03) while those for zidovudine were not significantly different (5.1 μM vs. 2.4 μM; Student's t-test, P = 0.5). Twelve (80%) of 15 isolates with codon 123 asp had IC_{90}s for didanosine ≥0.8 μM compared to 3 (27%) of 11 with codon 123 glu or gly (Fisher's exact test, P = 0.015).

DISCUSSION

This study examined the relationship between RT genotype and in vitro antiretroviral susceptibility phenotype of the HIV isolates obtained from patients with advanced HIV disease who were treated with didanosine



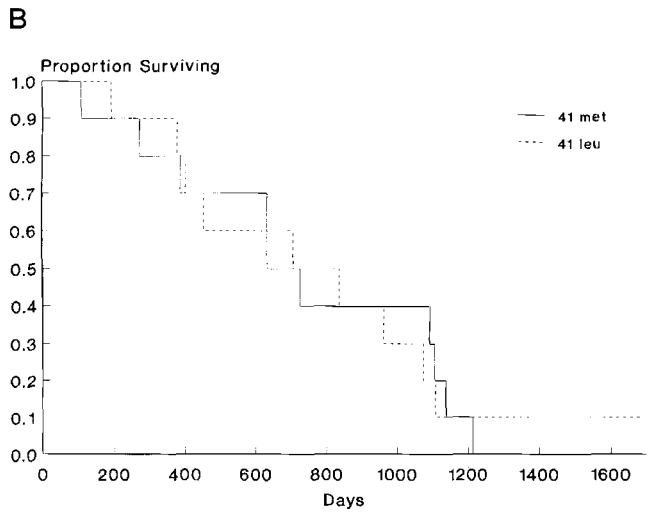


Fig. 3. Survival in patients with zidovudine resistance mutations in the pre-treatment isolate. **A:** Wild-type (n = 6) or mutant (n = 14) sequences at codon 215. **B:** Wild-type (n = 10) or mutant (n = 10) at codon 41.

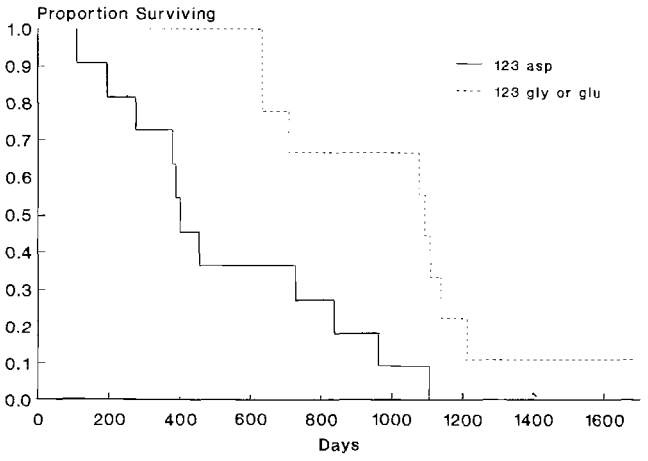


Fig. 4. Survival in patients (n = 20) with sequence variation at codon 123.

after treatment failure or intolerance to zidovudine. The purpose was to identify markers which correlate with clinical and virologic response to didanosine therapy, novel RT gene mutations associated with resistance, or mutations otherwise predictive of outcome in this patient population. No clear relationship between virologic response and in vitro susceptibility was apparent. The lack of an observed correlation could be due to the imprecision of the tools used to measure either susceptibility to didanosine or the virologic response. Alternately, in patients with very advanced immunodeficiency, variables such as compliance with treatment, drug disposition, and intercurrent illness could be more important determinants of virologic and clinical outcome.

Using a cut-off value of 1 μM, the antiretroviral susceptibility assay used in the study was able to differentiate most zidovudine susceptible from resistant strains, as determined by genotypic analysis showing amino acid substitutions at codon 215 plus one or more additional resistance mutations. However, no clear cut-off for didanosine resistance could be established by correlation to virologic response or RT genotype. Although strains acquiring didanosine resistance mutations had changes in IC_{90} values, these values were not clearly higher than values found in some isolates lacking known resistance mutations. Among paired isolates, most changes in susceptibility of fivefold or more were associated with acquisition or loss of known antiretroviral resistance mutations. Other isolate pairs were less variable. The inability of the antiviral susceptibility assay to identify a threshold value for sensitivity to didanosine may be due to its relative insensitivity to changes of the magnitude being measured and the relatively broad range of sensitivities exhibited by isolates from previously untreated patients. Combinations of zidovudine resistance mutations may alter susceptibility by 100-fold or more [Kellam et al., 1992], and such changes are readily identified. The RT gene mutation leu 74 val produces only a 7–26-fold decrease in susceptibility to didanosine [St. Clair et al., 1991], and other described didanosine resistance mutations result in more modest decreases [Zhang et al., 1994; Gu et al., 1992, 1994; Lacey and Larder 1994; Gao et al., 1992]. The relationship between the IC_{90} values for zidovudine and didanosine is similar to that demonstrated in a previous report [Mayers et al. 1994] and suggests that additional factors, such as differences in growth characteristics among strains, may also have a modest influence on phenotypic susceptibility results in a PBMC-based assay. Other investigators using this type of assay system have also found a broad range of in vitro sensitivities of clinical HIV isolates and could not report a distinct threshold value associated with didanosine resistance [St. Clair et al., 1991; McLeod et al., 1992; Reichman et al., 1993]. The recombinant virus assay,

which introduces RT sequence variation from clinical HIV isolates into a cloned genetic background [Kellam and Larder, 1994], might help overcome the limitations of the PBMC-based assay. Future investigations should investigate the use of such assays or develop other more precise phenotypic susceptibility testing systems for didanosine.

In the previous study, two-thirds of patients with detectable viremia had a decline in serum virus titer after 2 months of didanosine treatment. The virologic response correlated with short-term clinical benefit manifesting as weight gain, but not with survival [Shepp and Ashraf, 1993]. More recently, quantitative determination of HIV RNA in plasma has been shown to correlate well with both the clinical benefit of and emergence of resistance to antiretroviral agents [O'Brien et al., 1996; Schuurman et al., *1995*] and with risk of progression to AIDS [Mellors et al., 1995]. Future investigations using this newer virologic tool could reveal more precise correlations with susceptibility phenotype and clinical outcome.

Development of genotypic resistance to didanosine, as indicated by the emergence of the leu 74 val mutation, was seen less often in this study than in a previous report [Kozal et al., 1994]. The reasons for this difference is not clear. Patients in the present study had lower baseline CD4 lymphocyte counts and many more were changed to didanosine due to zidovudine treatment failure. Therefore, these patients had more advanced immunodeficiency than those in the previous study, and might be expected to have a greater likelihood of developing resistance. However, the median duration of didanosine treatment was shorter in the current study. The lower rate of didanosine resistance may result from a shorter duration of treatment, which in turn may reflect poorer tolerance of the medication, reduced compliance with the prescribed regimen prior to discontinuation, and shorter survival. More advanced stage illness has been recognized as a risk factor for adverse effects during didanosine treatment [Schindzielorz et al., 1994].

Although mutation at codon 215 is a marker for zidovudine resistance and does not affect didanosine susceptibility, in the present study codon 215 mutations were found to be strongly associated with shorter survival during didanosine treatment. Studies in patients with less advanced HIV infection also have associated codon 215 mutations with more rapid CD4 lymphocyte depletion [Kozal et al., 1993] and with increased risk of death, especially when present in combination with mutation at codon 41 [Japour et al., 1995]. This study demonstrates that codon 215 mutations also are associated with poorer survival in patients with advanced HIV disease who are changed to didanosine because of intolerance or clinical failure during zidovudine therapy. However, codon 215 mutations were strongly associated with other factors affecting survival, and because of the small size of the study population it was not possible to determine if codon 215 mutation was an independent risk factor for death when other variables were controlled.

The observed association of codon 123 variation with survival and didanosine susceptibility phenotype was an unexpected result. Sequences encoding aspartic acid at codon 123 were strongly associated with other factors negatively affecting survival, including codon 215 mutations. Codon 123 sequence variability could play a role in the pathogenesis of HIV disease, possibly as a compensatory mutation enhancing the efficiency of a drug-resistant RT. Alternately, this mutation may be a marker for end-stage AIDS, which plays no pathogenic role, or the finding may merely be a statistical artifact resulting from analysis of multiple variables in a small sample size. The finding that isolates encoding aspartic acid at codon 123 have somewhat higher IC_{90} values for didanosine, but not zidovudine, suggests a possible role for sequence variability at this codon in determining responsiveness to didanosine treatment. However, this observation is confounded by the possibility that susceptibility testing may be subtly affected by factors such as difference in growth rates of strains obtained from patients with more advanced HIV disease. Larger, prospective or case-control studies will be needed to investigate a possible role for codon 123 variability as a pathophysiologic or prognostic factor in patients with advanced HIV disease, and the biological effect of sequence variability on didanosine resistance will need to be established by introduction of specific sequence changes using site-directed mutagenesis.

Another unusual finding was the appearance of codon 215 mutations during didanosine treatment in isolates from two patients with wild-type sequences in the pretreatment isolates. This observation confirms other recent reports of the emergence of zidovudine resistance mutations during therapy with other nucleoside RT inhibitors [Lin et al., 1994; Demeter et al., 1995]. Surreptitious zidovudine use cannot be completely excluded, but is an unlikely explanation due to the lack of hematologic abnormalities in the two patients.

Reversion of zidovudine resistance mutations was observed in the post-treatment isolates of two-thirds of patients withdrawn from zidovudine and treated with didanosine. However, only at colon 70 did a majority of mutant pre-treatment isolates undergo reversion, and no reversions at codons 67 and 215 were observed. Although patients with less advanced HIV disease who are switched to didanosine without evidence of zidovudine intolerance or treatment failure have been reported to lose codon 215 mutations more rapidly [Kozal et al., 1994], these findings are similar to the slow rates of reversion of zidovudine resistance mutations described by other investigators who have studied patients with advanced HIV disease [Smith et al., 1994; Boucher et al., 1993; Masquelier et al., 1995]. Only one isolate reverting at two codons had a substantial increase in susceptibility, and in no revertant isolate did the IC_{90} decline below 1 μM. The lack of improvement in zidovudine susceptibility may be attributable to the persistence of codon 215 mutations in post-treatment isolates and to the appearance of codon 215 mutations in two isolates. In contrast to previous findings [St. Clair et al., 1991], these results suggest that significant improvement in zidovudine

susceptibility is not a common effect of sequential treatment with didanosine after zidovudine.

Although previously described zidovudine resistance mutations at codon 215 were most prevalent, sequences encoding unusual amino acid substitutions were identified in several isolates. The isolate with a mixture of sequences ATC/ACC encoding isoleucine/threonine may represent an intermediate form on the path to the two step mutation TTC encoding phenylalanine. The patient from whom this isolate was obtained had been treated with zidovudine for only 2 months. This mutation has also been noted as a revertant in a patient withdrawn from zidovudine [Masquelier et al., 1995]. The sequence GAC encoding aspartic acid may represent a partial revertant from the mutation TAC encoding tyrosine. This patient had discontinued zidovudine 9 months before the isolate was obtained. The mutations encoding cysteine, glutamine and the mixture of tyrosine/histidine all occurred in patients with prolonged prior zidovudine use and without a prolonged interval between treatments, and appear to be unusual variants. All three were resistant to zidovudine in the susceptibility assay. However, studies employing site-directed mutagenesis will be required to establish the contribution of each of these mutations to zidovudine resistance.

Recent studies support the use of didanosine in combination with other antiretrovirals for treatment of HIV disease [Gazzard, 1996, Hammer et al., 1995]. However, resistance to any component of the combination may reduce its effectiveness. Many patients with advanced HIV disease have received prolonged prior therapy with zidovudine. Because neither reversion of zidovudine resistance nor emergence of recognizable didanosine resistance appears to occur rapidly during didanosine treatment, use of didanosine in combination with antiretroviral agents other than zidovudine may be advantageous in these patients. Future clinical trials should investigate such treatment combinations.

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